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(54) Title: THE PROTECTION OF EQUINES AGAINST STREPTOCOCCUS EQUI (57) Abstract A new bacterial vaccine to protect susceptible equine against <i>S. equi</i> which causes strangles. The vaccine stimulates a nasopharyngeal immune response in a susceptible equine through the presence of antibody activity in the nasopharyngeal mucus. The vaccine is a <i>S. equi</i> strain which contains an M protein fragment of 41,000 mw and is adapted for administration to equine either intranasally or orally as a vaccine. There is described a new strain of <i>S. equi</i> (709-27), a method of making and isolating useful vaccine strain of <i>S. equi</i> bacteria which stimulates an antibody response in the nasopharyngeal mucosa of the susceptible equine.		

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- 1 -

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TITLE

The Protection Of Equines Against Streptococcus Equi

BACKGROUND OF THE INVENTION

15 This invention relates to the immunization of equines against Streptococci equi. S. equi causes strangles, an acute upper respiratory tract disease of horses, characterized by fever, nasal discharge and abscess formation in the retropharyngeal and mandibular
20 lymph nodes. Horses that have been so infected in the field or experimentally infected with strangles and which do recover from strangles become highly resistant to reinfection. Moreover, only one antigenic type of S. equi has been observed in the field.

25 The above notwithstanding, vaccines prepared from bacterins of S. equi, or fractional extracts of the same, such as M protein-rich extracts, have been relatively ineffective to provide protection against S. equi in the field. This is true even though as far back as 1943 an
30 article entitled "Studies with Equine Streptococcus" published in the Australian Veterinary Journal at 19:62 by P. O. Bazeley, presented a broad-range study of the problem coupled with test results which Dr. Bazeley and other characterized as very hopeful. However, many years
35 have passed without an adequate or effective method or

- 2 -

1 means for protection of equines against strangles. One of
the problems with earlier experimentation in the field was
that scientists and researchers equated protection of the
horse against S. equi with stimulation of bactericidal
5 antibodies in the blood serum of the horse. In fact,
vaccine failure was not due to failure of vaccines to
stimulate bactericidal antibody in the serum, which it was
shown did not equate with protection against field or
experimental exposure to S. equi. In fact, it was dis-
10 covered that ponies recently recovered from experimentally
induced strangles were highly resistant to reinfection
before serum bactericidal activity could be detected.
Moreover, it was determined that the nasopharyngeal mucus
of resistant ponies contained major IgG and IgA antibody
15 activity against only one acid extract protein of about
41,000 molecular weight (mw), whereas serum antibodies had
a number of major specificities. These findings suggested
that successful vaccination requires stimulation of the
nasopharyngeal immune response.

20 The following publications have been made by the
inventor herein relating to this development:

- 1) Abstract No. 172 appearing Abstracts IXth,
Lancefield International Symposium on
Streptococci and Streptococcal Diseases, Fuji,
25 Japan, September 10, 1984;
- 2) Infection and Immunity, March 1985, Vol. 47,
No. 3, pages 623-628;
- 3) Infection and Immunity, April 1985, Vol. 48,
No. 1, pages 29-34.

30

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a graph with separate coordinates for
the IgA and IgG antibody titers in nasal washes of 14
ponies against days which have passed following immuni-
35 zation with S. equi 709-27. The antigens in the

3-

1 radioimmunoassay were acid extract and culture supernatant
(the native form) protein of S. equi.

Figure 2 is a graph of cumulative mortality
against days after challenge for groups of 40 mice vacci-
5 nated with live S. equi 709-27, or an acid extract of S.
equi 709-27 and a group of control non-immunized mice.
All mice were challenged with 5×10^{-7} -CFU virulent S.
equi CF32. The information of Fig. 2 is important because
it shows that S. equi 709-27 carries the intact M protein,
10 similar to that of the parent S. equi CF32.

Figure 3 is an immunoblot showing proteins (SDS
PAGE), S. equi and S. zooepidemicus recognized by IgG and
IgA in nasopharyngeal mucus and in serum of a pony follow-
ing intranasal vaccination with S. equi 709-27. The blots
15 were washed in monospecific antisera against equine IgA or
IgG following treatment with nasopharyngeal mucus or serum.

Tracks: A - Acid extract of S. zooepidemicus

B - Acid extract of S. Equi (CF32)

C - Culture supernatant protein of S. equi (CF32)

20 Antigens of S. zooepidemicus were included because
most horses carry this organism in the nasopharynx and
therefore are stimulated to make antibodies to its
proteins, some of which are common to S. equi.

The measurement technique described in the
25 Figures are similar to those discussed in the following
publications:

a) Infection and Immunity Vol. 47, No. 3 pages
623-628 (March 1985);

30 b) Infection and Immunity Vol. 48, No. 1 pages
29-34 (April 1985).

DESCRIPTION OF THE INVENTION

The present invention teaches how to stimulate
the nasopharyngeal immune response, for example using a
35 bacterial clone derived from a highly virulent strain of
S. equi known as S. equi CF32 which is on deposit at the

- 4 -

1 American Type Culture Collection, (A.T.C.C. No. 53185)
Rockville, Maryland, and available to the public as of the
time this patent application is issued. CF32 produces
large (1-3 mm) transparent, mucoid colonies that tend to
5 flow together and are surrounded by a wide zone (5-10 mm)
of beta hemolysis. Also on deposit with the American Type
Culture Collection is a derivative of S. equi CF32 which
has been rendered avirulent according to the teachings of
the present invention. The avirulent derivative S. equi
10 bacterium is known as Cornell S. equi 709-27 and will be
available through the A.T.C.C. under A.T.C.C. No. 53186
when this patent application issues as a U.S. Patent.
Cornell 709-27 produces a small (0.5 mm in diameter white,
convex smooth surfaced colony surrounded by a narrow (1
15 mm) zone of beta hemolysis.

This invention relates to an equine vaccine
against S. equi caused strangles in an equine, which
vaccine stimulates a nasopharyngeal immuno response in a
strangles susceptible equine and which vaccine comprises
20 an avirulent strain of S. equi formed by mutating a
virulent strangles causing S. equi strain to render it
avirulent while retaining thereon protein which provides
an acid extract M protein fragment with a molecular weight
of about 41,000 which stimulates an immunological response
25 to IgG and IgA antibody similar to that in the nasopharyn-
geal mucus of an equine recovered from S. equi caused
strangles.

The vaccine of the invention is not strain
specific. Only one antigenic type of S. equi has been
30 observed in the field. Thus, the method of the invention
can be applied to any virulent strangles causing S. equi
strain.

The virulent S. equi strain can be rendered
avirulent in any manner so long as the resultant avirulent
35 S. equi strain retains the M protein fragment having a

- 5 -

1 molecular weight of about 41,000 which stimulates an
immunological response similar to that in the nasopharyn-
geal mucus of an equine recovered from S. equi caused
strangles. The presently preferred method is deliberately
5 induced mutagenesis for example by the use of chemicals or
radiation. Particularly useful is chemical mutagenesis
for example through the use of nitrosoguanidine. (See
Chapter 13, Gene Mutation, Manual of Methods of General
Bacteriology, American Society for Microbiology,
10 Washington, D.C. 1981).

For the purposes of characterizing the vaccine of
the invention through radio-immunoassay or immunoblotting
assay the acid extract protein is isolated following
techniques described in a publication by R.C. Lancefield
15 entitled "The Antigenic Complex of Streptococcus
Hemolyticus I Demonstration of a Type Specific Substance
in Extracts of Streptococcus Hemolyticus" J. Exp. Med.
47:91.

For the purpose of further characterizing the
20 vaccine of the invention protein molecular weight is
determined by SDS - PAGE Electrophoresis and the use of
molecular weight standards.

In accordance with the teachings of the present
invention a successful vaccine against S. equi requires
25 stimulation of the nasopharyngeal immune response in a
susceptible equine by intranasal or oral inoculation.
Antibody activity in the nasopharyngeal mucus correlates
with protection against strangles, and antibody activity
in the blood serum is of less significance.

30 M-protein-rich extracts were relatively ineffec-
tive because they did not stimulate a nasopharyngeal
immune response of the susceptible equine, although they
were effective in producing an immune response in the
blood serum of the animal. In order to stimulate the
35 required response the present invention teaches a method
of making avirulent S. equi bacteria which may be used as

- 6 -

1 a vaccine and applied either intranasally or orally and
produces major IgG and IgA antibody responses in the
nasopharyngeal mucus of the susceptible equine. The
avirulent strain of S. equi (Cornell 709-27) is such a
5 bacteria. Hereinafter that especially made bacteria is
called S. equi (Cornell 709-27).

Method of Producing an Effective Avirulent
Vaccine Strain of S. equi

10 The strain of S. equi (Cornell 709-27) avirulent
for mice and ponies was obtained in the following manner:
the starting bacteria, S. equi CF32 was subjected to
nitrosoguanidine mutagenesis following the teachings set
out in an article by Carlton, B.C. and Brown, B.J. (1981)
15 in Manual of Methods for General Bacteriology. (Eds. P.
Gerhardt, et al.) American Society for Microbiology,
Washington, D.C., p. 226. Modification of the procedure
set forth in the first column of page 226 was undertaken.
Specifically, Todd Hewitt broth was used throughout the
20 procedures as a growth medium. Nonencapsulated colonies
were screened for loss of virulence by intraperitoneal
inoculation of mice (ICR). The strains which did not kill
mice were considered positive strains. The positive mouse
strains were then used to vaccinate mice by the intraperi-
25 toneal route to determine their protective quality. Those
strains which were protective of mice were inoculated
intranasally into horses. Finally, as described herein, a
derived strain of S. equi 709-27 was found to be avirulent
in a dose of 3×10^9 CFU, and efficacious as a vaccine
30 against S. equi in susceptible equine when it was intra-
nasally or orally inoculated in the equine. Moreover, the
positive strain which also protected equines tested for
the presence of the 41k fragment of the M protein by
immunoblotting. The identifying number for that strain is
35 S. equi 709-27.

- 7 -

1 An acid extract of strain S. equi (Cornell 709-27)
 was shown by immunoblotting to carry the same immunolog-
 ically reactive proteins as the parent S. equi strain
 (CF32). The immunoblotting procedure used was similar to
 5 that used in a scientific article entitled "Infection and
 Immunity" Vol. 48, No. 1 pages 29-34 (April 1985).

Equine Immunization and Challenge

10 The S. equi (Cornell 709-27) was then tested for
 efficacy as a vaccine against experimental S. equi
 infection in equine. The following table depicts that
 testing.

Table 1. Resistance of Ponies to Intranasal Challenge
 with Streptococcus equi Following Intranasal
 15 Immunization with the Avirulent Strain of
S. equi 709-27.

Treatment (vaccinate with 20 <u>S. equi</u> 709-27)	Challenge (CFU Virulent <u>S. equi</u> CF32)	No. Ponies	No.. Resistant
3 x 10 ⁹	5 x 10 ⁸	14	14
Day 0 and Day 30	Day 59		
25 Contact Controls	"	2	2
Isolation Controls	"	6	0*

30 *All controls developed acute strangles within 4 days of
 challenge.

Fourteen yearling ponies raised in isolation and
 never exposed to S. equi were given an atomized suspension
 (intranasally) of an 18 hour culture (3 10⁹ CFU) in Todd
 Hewitt broth of 709-27. A repeat inoculation was given 29
 35 days later. Ponies were challenged intranasally 30 days

- 8 -

1 later with 5×10^8 CFU of an overnight culture of S. equi CF32. Cultures were administered by means of a nasal atomizer (Model #281, Devilbiss Co., Somerset, PA).

5 Six non-vaccinated ponies housed separately from the vaccinated group and 2 contact control ponies were also challenged with the same CF32 inoculum. All of the immunized ponies and the 2 contact control ponies were resistant to S. equi when challenged, but all of the isolation controls developed acute strangles within 4 days
10 of challenge.

In addition about 800 horses on farms with endemic S. equi infection problems were experimentally intranasally or orally vaccinated with S. equi 709-27 to date and only two horses have developed strangles. The
15 expected occurrence of strangles on those farms based on the experience of the three previous years, is such that one would have predicted the occurrence of strangles in the range of 40% of the horses.

When using the teachings of the present invention
20 to vaccinate horses against S. equi the results of oral inoculation appeared to be comparable with intranasal inoculation with the same dose. The vaccine dose (number of organisms) used in the vaccination described herein was 100 times greater than the number of organisms of a wild
25 virulent strain of S. equi (CF32), which would be expected to cause disease in a normal equine. However, a commercial S. equi vaccine program would utilize dosage levels which were determined by consultation between the manufacturer and the appropriate governmental authorities.

30 Freezing or freeze drying does not adversely affect the vaccine. These procedures can therefore be used in mass production and distribution of the vaccine.

The vaccine has been entirely without side effects in adult animals, but a low (~5%) incidence of
35 submandibular abscesses has been observed on one occasion in 3-month foals. This adverse reaction occurred when a

- 9 -

1 very heavy dose of vaccine was administered in an effort
to obtain consistent seroconversion in the blood serum of
the inoculated equine. As stated elsewhere, it is
nasopharyngeal mucus of the susceptible equine that
5 contains antibodies involved in immunological protection.

Antibody Assays - Figure 1

IgA and IgG antibodies to the proteins of S. equi
(CF32) were assayed in sera and nasal washes collected
10 before, during, and after vaccination and challenge.
Assays were performed by solid phase radioimmunoassay as
described in an article entitled "Immunochemical Quan-
titation of Antigens by Single Radial Immunodiffusion"
by G. Mancini, A.O. Carbonara and J.H. Heremans in
15 Immunochemistry 2: pages 235-254. Wells were coated with
acid extract (AE) or culture supernatant (CS) protein of
S. equi.

IgA and IgG antibody responses to acid extract
and culture supernatant proteins of S. equi were observed
20 in nasal washes from all vaccinated animals (Figure 1).
Serum antibody responses were also observed, but they were
inconsistent. Contact control ponies showed nasal and
serum antibody conversion at the same time - an indication
that transmission of the vaccine strain had occurred in
25 the group. Principal and contact control ponies were
resistant to challenge with virulent S. equi whereas
non-vaccinated ponies developed typical strangles within 4
days of challenge (Table 1).

30 Mouse Immunization and Challenge - Figure 2

The mouse has historically been the model for the
immunology of S. equi infection. Accordingly, as a
parallel test of efficacy, adult ICR mice were immunized
subcutaneously with hydroxyapatite purified protein of an
35 acid extract of S. equi 709-27. Reference is made to an
article by Vosti, K.L. Journal of Med. Microbiol. 11:453

-10-

1 (1978). Protein was absorbed to aluminum hydroxide and
administered in two subcutaneous doses of 50 µg 21 days
apart. All mice, including a group of non-vaccinated
controls, were later challenged with virulent S. equi ($5 \times$
5 10^7 CFU) given intraperitoneally. Mouse mortality was
recorded for 7 days following challenge. The difference
in mortality between the control and vaccinated groups was
highly significant using the Chi square analysis used in
statistics. The mice immunized either with an acid
10 extract or live cells of S. equi 709-27 showed a signifi-
cant protective response (probability $\leq .01$) as compared
with non-vaccinated controls (Figure 2). This result
suggested that S. equi 709-27 retained the protective M
antigen of S. equi.

15 Notwithstanding the fact that it is not virulent,
an acid extract of S. equi 709-27 was shown by immuno-
blotting to carry the same immunologically reactive
proteins as the parent S. equi strain.

20 Fig. 3 Immunoblotting Showing Proteins Recognized
by Mucosal and Serum Antibodies

The immunologically reactive proteins in an acid
extract and culture supernatant of S. equi and an acid
extract of S. zooepidemicus were distinguished on nitro-
25 cellulose blots of SDS - PAGE gels. Blots were treated
with sera or nasopharyngeal mucus collected when ponies
were killed 7 days after challenge. A scientific article
entitled "Infection and Immunity" Vol. 47, No. 3 pages
623-628 (March 1985) describes the technique used.

30 Immunoblotting revealed that IgA and IgG
antibodies in nasopharyngeal mucus of vaccinated animals
were directed mainly against a 41k M protein fragment,
whereas serum antibodies had a much broader spectrum of
activity, a finding noted previously in ponies following
35 recovery from experimentally induced strangles. Since an
antibody response to the 41,000 mw M protein fragment is a

- 11 -

1 constant feature of the nasopharyngeal immune response of
resistant horses, it is an important protective antigen.

The antibody response is also specific to S. equi
because similarly reactive proteins of S. zooepidemicus
5 could not be detected on the immunoblot (Track A). Other
studies have indicated that antibodies in strongly
bactericidal sera react strongly with M protein fragments
of about 29,000 and 37,000 molecular weight. A hypothesis
to explain the different molecular weights of the M
10 protein fragments of S. equi recognized by serum and
nasopharyngeal antibody is that the portion or region of
the M protein molecule of S. equi important in the
nasopharyngeal response, differs from that involved in the
stimulation of bactericidal antibody in serum.

15 In summary, the present invention teaches a new
and improved bacterial vaccine to protect susceptible
equine against S. equi which causes strangles. The
vaccine stimulates a nasopharyngeal immune response in a
susceptible equine through the presence of antibody in the
nasopharyngeal mucus. The vaccine is a S. equi strain
20 which contains an M-protein fragment of 41,000 mw and is
adapted for administration to equine either intranasally
or orally as a vaccine. The teachings of the present
invention include: a new strain of S. equi (709-27), a
25 method of making and isolating useful vaccine strain of S.
equi bacteria, and which stimulates an antibody response
in the nasopharyngeal mucosa of the susceptible equine.

Accordingly, it is to be understood that the
embodiments of the invention herein described are merely
30 illustrative of the application of the principles of the
invention. Reference herein to details of the illustrated
embodiments are not intended to limit the scope of the
claims which themselves recite those features regarded as
essential to the invention.

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- 12 -

I CLAIM:

1. A vaccine for protecting equines against S. equi caused strangles which comprises an avirulent strain of S. equi which stimulates an antibody response in the nasopharyngeal mucosa of the susceptible equine.

2. A vaccine as in Claim 1 against S. equi caused strangles in an equine, which vaccine stimulates a nasopharyngeal S. equi antibody response in a strangles susceptible equine and which vaccine comprises an avirulent strain of S. equi formed by mutating virulent strangles causing S. equi strain to render it avirulent while retaining thereon protein which provides an M protein fragment with a molecular weight of about 41,000 which stimulates an immunological response in the form of IgG and IgA antibodies in the nasopharyngeal mucus of an equine similar to that found in an equine which has recovered from S. equi caused strangles.

3. The vaccine of Claim 1 wherein the strain avirulent S. equi is nonencapsulated and includes an M-protein fragment with a molecular weight of about 41,000.

4. The vaccine of Claim 3 wherein the strain of avirulent S. equi is S. equi 709-27.

5. The vaccine of Claim 1 wherein the strain of avirulent S. equi includes an M-protein fragment with a molecular weight of about 41,000 and which can be inoculated intranasally or orally.

6. The vaccine of Claim 5 wherein the strain avirulent S. equi is S. equi 709-27.

7. A method of protecting equines against avirulent S. equi which comprises inoculating an equine either intranasally or orally with a strain of avirulent S. equi which stimulates a nasopharyngeal antibody response in the nasopharyngeal mucosa of a susceptible equine.

- 13 -

1 8. The method of Claim 7 wherein the strain of S. equi is both avirulent and carries at least M-protein fragments of about 41,000 mw.

5 9. The method of Claim 8 wherein the strain avirulent S. equi is S. equi 709-27.

10 10. A vaccine for protecting equines against S. equi which comprises an avirulent strain of S. equi known as S. equi 709-27 which can be inoculated intranasally or orally in the susceptible equine.

15 11. A method of making a strain of S. equi which is avirulent for equines comprising of the following steps:

1. subjecting a virulent strain of S. equi to mutagenesis;

2. selecting a resulting bacterium which provides an M protein fragment having a molecular weight of 41,000, which bacterium produces an S. equi antibody response in the nasopharyngeal mucus of an S. equi susceptible equine.

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SUBSTITUTE SHEET

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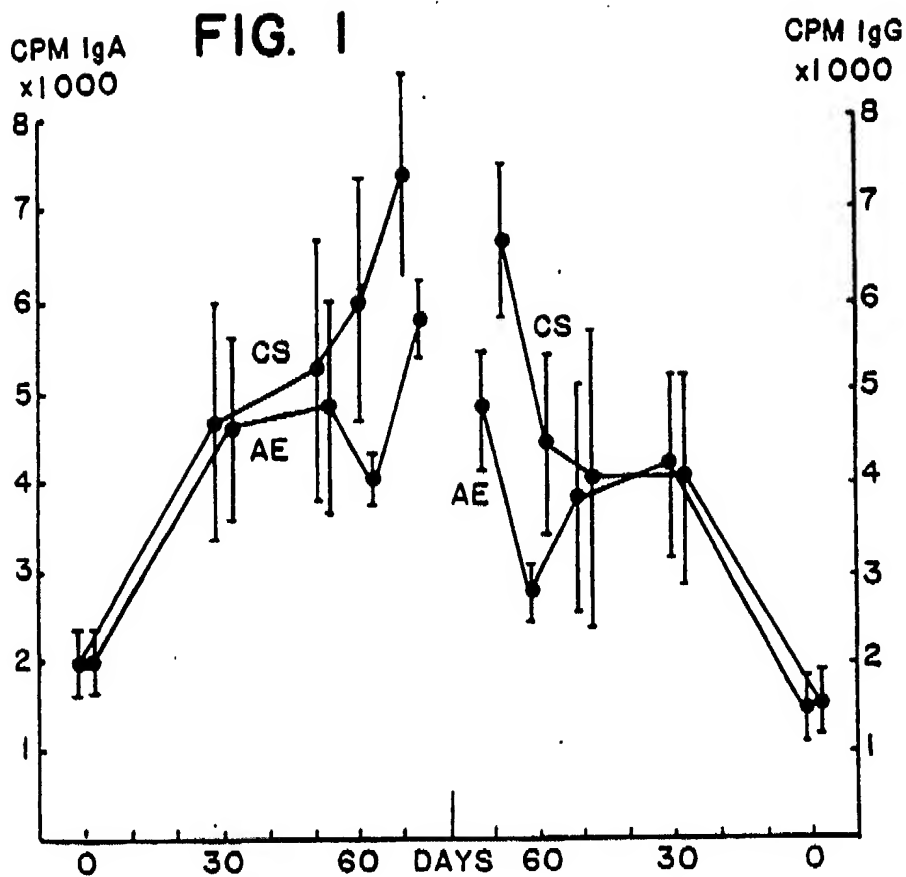


FIG. 3

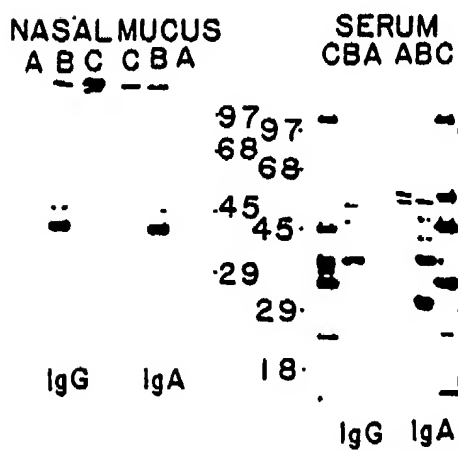
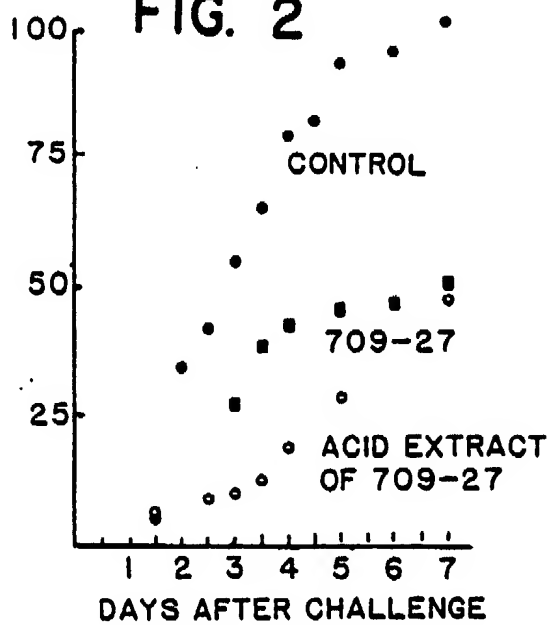
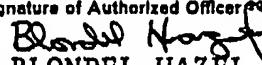


FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US86/01460**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC4: A61K 39/09; C12N 15/00; 1/20 U.S.: 424/92,93; 435/172.1, 253		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	424/92, 93; 435/172.1, 253	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
ONLINE COMPUTER SEARCH CHEMICAL ABSTRACTS 1967-1986; BIOSIS 1967-1986. SEARCH TERMS: STREPTOCOCCUS EQUI, ATTENUATED STRAINS AND VACCINES.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A, P	US, A, 4,582,798 (BROWN) 15 April 1986. See entire document.	1-10
A	Infection and Immunity, Volume 48, No. 1, issued 1985 (U.S.A.), J. F. Timoney, "Immunologically Reactive Proteins of Streptococcus equi". See pages 29-34.	1-10
A	Infection and Immunity, Volume 47, No. 3, issued 1985 (U.S.A.), Jorge E. Galan, "Mucosal Nasopharyngeal Immune Response of Horses to Protein Antigens of Streptococcus equi". See pages 623-628.	1-10
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁶ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
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10 September 1986	23 SEP 1986	
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